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Critical Role of TLR2 and TLR4 in Autoantibody Production and Glomerulonephritis in lpr Mutation-Induced Mouse Lupus

Aurelia Lartigue,²* Natacha Colliou,²* Sébastien Calbo,,* Arnault François,† Serge Jacquot,*,‡ Christophe Arnoult,*, Francois Tron,*, Daniele Gilbert,* and Philippe Musette³*§

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by pathogenic autoantibodies directed against nuclear Ags and immune complex deposits in damaged organs. Environmental factors have been thought to play a role in the onset of the disease. The recognition of these factors is mediated by TLRs, in particular TLR2 and TLR4 which bind pathogen-associated molecular patterns of Gram⁺ and Gram⁻ bacteria, respectively. We attempted to determine the role of these TLRs in SLE by creating TLR2- or TLR4-deficient C57BL/6lpr/lpr mice. These mice developed a less severe disease and fewer immunological alterations. Indeed, in C57BL/6lpr/lpr-TLR2 or -TLR4-deficient mice, glomerular IgG deposits and mesangial cell proliferation were dramatically decreased and antinuclear, anti-dsDNA, and anti-cardiolipin autoantibody titers were significantly reduced. However, the response against nucleosome remained unaffected, indicating a role of TLR2 and TLR4 in the production of Abs directed against only certain categories of SLE-related autoantigens. Analysis of B cell phenotype showed a significant reduction of marginal zone B cells, particularly in C57BL/6lpr/lpr-TLR4-deficient mice, suggesting an important role of TLR4 in the sustained activation of these cells likely involved in autoantibody production. Interestingly, the lack of TLR4 also affected the production of cytokines involved in the development of lupus disease. The Journal of Immunology, 2009, 183: 0000–0000.

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ystemic lupus erythematosus (SLE) is a nonorgan-specific autoimmune disease characterized by the production of autoantibodies directed against self-nuclear Ags, in particular dsDNA and nucleosomes, (1) that may induce glomerulonephritis through immune complex deposits (2). SLE is characterized by an intrinsic hyperactivity of B cells (3–5) and by IFN-α/β-induced B and T cell activation (6, 7). SLE is thought to result from interaction between genetic and environmental factors (8). The role of the latter is suggested by the observation that in human lupus, the onset of the disease and clinical flares can be associated with viral infections (9, 10) and, in lupus-prone mice, repeated injections of bacteria-derived components increase the severity of the disease (11). One of the mechanisms whereby bacteria and viruses can participate in autoimmune disorders is their interaction with TLRs, which are receptors for various microbial components referred to as pathogen-associated molecular patterns (12, 13). Each TLR has evolved to recognize a specific set of these molecular patterns, including components of bacterial cell walls for TLR2 and 4 and nucleic acids such as dsRNA, ssRNA, and unmethylated CpG dinucleotide-containing DNA for TLR3, 7, and 9, respectively.

Most TLRs are expressed by immune cells, including dendritic cells, B lymphocytes, and some categories of T lymphocytes (14). However, differences in TLR expression exist between humans and mice, particularly in B cells, since mouse, but not human, naive B cells express TLR4. In this respect, it has long been known that LPS induces polyclonal activation of mouse B cells, leading to their differentiation into Ab-secreting plasma cells (15). It now appears that this property is related to the presence of TLR4 on mouse B cells.

TLR2 and TLR4 are also expressed on regulatory T cells (Tregs) (16) and their stimulation enhances survival and proliferation of these cells (17). However, in the presence of TLR2 ligands, the suppressive function of Tregs is temporally abrogated (17). TLR4 ligation could also overcome the suppressive effect of Tregs but, in this case, it requires the presence of dendritic cells and possibly the production of IL-6 (18).

The involvement of TLRs in the onset of autoimmune diseases is currently a matter of intensive study. In lupus, several reports have focused on the role of TLRs which recognize nucleic acids including TLR3, 7, and 9 (19, 20) and demonstrated that their engagement participates in the induction of antinuclear autoantibodies. Recently, using the C57BL/6lpr/lpr model, we demonstrated that TLR9 is basically required for production of anti-nucleosome Abs (21). Significantly, TLR9-deficient mice presented a more severe disease, suggesting that TLR9 engagement may have a protective role, possibly through the induction of autoantibodies participating in the clearance of apoptotic cell products known to initiate and propagate the disease (20).

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Abbreviations used in this paper: SLE, systemic lupus erythematosus; MZ, marginal zone; TI, T cell independent; Treg, regulatory T cell; HSP, heat shock protein; WT, wild type; RF, rheumatoid factor; MZ, marginal zone; BLyS, B lymphocyte stimulator; TACI, transmembrane activator calcium modulator and cyclophilin ligand; ANA, antinuclear Ab; CL, cardiolipin.

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The roles of TLR2 and TLR4 in systemic autoimmune diseases are still poorly documented. TLR2 serves as a receptor for peptidoglycan and lipoproteins of Gram-positive bacteria (22) and TLR4 as a receptor for Gram-negative LPS (23). These receptors also bind viral-envelope proteins, different endogenous ligands (13), and molecules derived from damaged cells as heat shock protein (HSP) 60 (24, 25). In this respect, it was shown that TLR2 signaling is important for the onset of streptococcal cell wall-induced arthritis (22) and that TLR4 participates in the control of the arthritis induced by K/BxN serum transfer (25). Recently, using a model of transgenic mice for a chaperone molecule (gp96), it has been demonstrated that an increased TLR4 signaling could induce a lupus-like autoimmune glomerulonephritis (26).

In this study, we addressed the question of the role of TLR2 and TLR4 in the development of a spontaneous lupus disease. For this purpose, we generated TLR2- and TLR4-deficient lupus prone mice by crossing C57BL/6 mice (B6) bearing homozygous FAS<sup>129</sup>/<sup>129</sup> mutation, that develop a moderate type of lupus related to Fas deficiency (27), with B6-Tlr2<sup>−/−</sup> or B6-Tlr4<sup>−/−</sup> mice. After RBC lysis, splenocytes were counted, adjusted at 10<sup>7</sup> cells/ml, and the phenotype of the disease, autoantibody production, and renal injury. Our data show that deficiency in TLR4 and, at a lesser extent in TLR2, down-regulates production of autoantibodies and attenuates the development of renal injuries and thus exerts a protective role in this strain of lupus-prone mice.

### Materials and Methods

#### Mice

B6 mice bearing homozygous FAS<sup>129</sup>/<sup>129</sup> mutation (B6<sup>129</sup>/<sup>129</sup>) and B6-Tlr2<sup>−/−</sup> or B6-Tlr4<sup>−/−</sup> mice were obtained from the Centre de Développement des Technologies Avancées and bred in our animal facilities. B6-Tlr2<sup>−/−</sup> and B6-Tlr4<sup>−/−</sup> mice were obtained from breeding B6<sup>129</sup>/<sup>129</sup> with B6-Tlr2<sup>−/−</sup> and B6-Tlr4<sup>−/−</sup> mice, respectively. B6<sup>129</sup>/<sup>129</sup> mice were used as controls. All mice were used in accordance with animal care and use regional committee procedures. To detect transgenes, genomic DNA was analyzed by PCR to identify a 178-bp wild-type (WT) and a 228-bp mutant fragment using TLR2-specific primers (5<sup>′</sup>-AGTACAGG, 5<sup>′</sup>-TCGACCTCGATCAACAGGAGAAG, 5<sup>′</sup>-CAAATTTTATTTGTTGCGACACCA), a 420-bp WT and a 320-bp mutant fragment using TLR4-specific primers (primer TLR4 3<sup>′</sup> 5<sup>′</sup>-TGTTGGGTGGTTTGTTCGGATCCGTCGG). PCR was performed for 35 cycles at 94°C for 30 s, 60°C for 115 s for (TLR4 and LPR) or 55°C for 30 s (TLR2), 72°C for 45 s for (LPR and TLR2), or 105 s (for TLR4). Taq polymerase thermoprime plus was obtained from Abgene.

#### Flow cytometry analysis and mAbs

After RBC lysis, splenocytes were counted, adjusted at 10<sup>7</sup> cells/ml, and 100 µl was incubated for 20 min on ice with optimal amounts of FITC-, PE-, PerCP-Cy5.5-, or PE-Cy7-conjugated primary Abs diluted in PBS. The following Abs were obtained, based from BD PharMingen, were used to assess B cell subsets: CD19, CD21/CD35, CD23 (FceRII), CD138 (Syndecan-I), anti-IgD, and anti-IgM. Data acquisition was performed on a FC 500 flow cytometer (Beckman Coulter) and analyzed with CXP software.

#### Dosage of cytokines

Twenty-five microliters of plasma was analyzed on a mouse cytokine/chemokine MILLIPLEX map (Millipore). Cytokines measured included IL-6, IL-10, IL-12, IL-13, and IFN-γ. A broad sensitivity range of standards ranging from 3.2 to 10,000 pg/ml was used. Concentration of cytokine in a sample was quantified using a standard curve. A 4-cubic spline method expression was performed to derive an equation that was used to predict the concentration of the samples.

#### Western blotting

Escherichia coli and Staphylococcus aureus cell pellets were suspended in a lysis buffer composed of 40 mM Tris-HCl (pH 8), 100 mM NaCl, 5 mM sodium-EDTA, 5 U/ml lysostaphin, 250 µg/ml lysozyme, 25 µg/ml DNase I, and protease inhibitors. After incubation for 1 h at 37°C, the suspension was sonicated at 40% for 1 min and centrifuged at 8000 × g for 20 min at 4°C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with mouse sera dilute 1/100. Bound IgM were detected with alkaline phosphatase-conjugated goat anti-mouse IgM (Rockland) and revealed with NBT/5-bromo-4-chloro-3-indolyl phosphate substrate (Roche).

#### Antinuclear Ab (ANA) test

For indirect immunofluorescence assay on HEP-2 cells (BMD), sera were diluted 1/100 and incubated for 1 h. After washing, bound IgGs were detected using a FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) or biotin-labeled anti-mouse IgG subclasses followed by FITC-streptavidin binding (Invitrogen and Caltag Laboratories). An index was determined based on fluorescence intensity (0, negative; 1, trace; 2, low; 3, intermediate; and 4, strong).

#### ELISAs

Anti-dsDNA and anti-nucleosome Abs were detected by ELISAs using plasmid-dsDNA precoated plates (Phadia) or DNA-stripped nucleosome (Euroimmun-BioAdvance) as the Ags on the solid phase. For antinuclear Ab, antissDNA Ab, rheumatoid factor (RF), and dosage of total mouse IgG/IgM and IgG subclasses detection, plates were incubated, respectively, with 10 µg/ml bovine heart CL, 1 µg/ml cal thymus ssDNA (Sigma-Aldrich), 10 µg/ml rabbit IgG (Jackson ImmunoResearch Laboratories), and 1 µg/ml goat F(ab′)<sub>2</sub> anti-IgG and -IgM (H + L; Caltag Laboratories). Mouse sera were diluted 1/4000 for Ig dosage and incubated for 2 h at room temperature. Bound IgG or IgM was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc-specific), anti-mouse IgM (µ specific; Rockland), or anti-IgG subclasses (Invitrogen and Caltag Laboratories), revealed with p-nitrophenyl phosphate (Sigma-Aldrich) and read at OD 405 nm.

#### Histopathology

Mice were analyzed at 6 mo of age and renal examination was performed. Half kidneys were fixed in 4% neutral formaldehyde, sectioned, and stained with trichrome green. We analyzed the presence of interstitial lymphoid infiltration and glomerulonephritis including mesangial cell proliferation. A nephritis index was determined based on the mesangial cell proliferation (0, <3 cells/glomeruli; 1, mild with 3–10 cells/glomeruli; 2, moderate with 10–15 cells/glomeruli; and 3, severe with >15 cells/glomeruli). Frozen kidney sections were stained with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) or anti-mouse IgG subclass (Caltag Laboratories). The topography and intensity of glomerular Ig deposits were assessed by a renal pathologist (A.F.).

#### Statistical analyses

All experiments were performed using at least three different cultures or animals in independent experiments. Statistical comparisons were performed using the Mann-Whitney U test. A value of p <0.05 was considered significant.

#### Results

**Clinical signs of SLE in B6<sup>129</sup>-Tlr2<sup>−/−</sup> and B6<sup>129</sup>-Tlr4<sup>−/−</sup> mice**

The phenotype of B6<sup>129</sup>-Tlr2<sup>−/−</sup> and B6<sup>129</sup>-Tlr4<sup>−/−</sup> mice was analyzed from birth to 6 mo of age. The most apparent clinical signs of lpr-induced SLE were splenomegaly and generalized lymphadenopathy due to an elevated number of activated CD4 T cells, CD4<sup>+</sup>CD8<sup>+</sup> double-negative T cells, and B1a cells (28). Spleen weights were lower in B6<sup>129</sup>-Tlr2<sup>−/−</sup> (n = 5; mean = 135.8 ± 45.8 mg; p < 0.001) and B6<sup>129</sup>-Tlr4<sup>−/−</sup> (n = 9; mean = 149.3 ± 91.9 mg; p = 0.035) than in B6<sup>129</sup>-Tlr2<sup>−/−</sup> (n = 5; mean = 328.3 ± 165.8 mg) (Fig. 1A). Likewise, cervical lymph node weights from B6<sup>129</sup>-Tlr2<sup>−/−</sup> and B6<sup>129</sup>-Tlr4<sup>−/−</sup> (mean = 5.2 ± 4 mg; 73.1 ± 77.8 mg) were lower than those obtained from B6<sup>129</sup> mice (209.8 ± 159.4 mg; p = 0.0003; p = 0.07) (Fig. 1B).

When the survival rate of B6<sup>129</sup>-Tlr2<sup>−/−</sup> and B6<sup>129</sup>-Tlr4<sup>−/−</sup> mice was compared with that of B6<sup>129</sup> mice up to 6 mo of age, no difference could be observed.
Kidney disease in TLR2- and TLR4-deficient B6lpr/lpr mice

Histological and immunochemical analyses of kidneys of 6-month-old mice were performed. Mesangial proliferation was analyzed and a nephritis index score was determined from 0 to 3. The rate of mesangial cell proliferation was decreased in both B6lpr/lpr TLR-deficient mice compared with B6lpr/lpr. Indeed, three of five B6lpr/lpr-Tlr2−/− mice (mean of score = 0.6 ± 0.54) and 5 of 9 B6lpr/lpr-Tlr4−/− mice (0.55 ± 0.5; p = 0.043) in comparison to 12 of 13 B6lpr/lpr mice (1 ± 0.4) showed mesangial cell proliferation (Fig. 2). IgG deposits were exclusively present in the mesangial areas (Fig. 3A) and their intensity, graded from 0 to 4 as described elsewhere (29), was significantly lower particularly in B6lpr/lpr-Tlr4−/− (mean of intensity score = 1.33 ± 0.5) vs B6lpr/lpr mice (3 ± 0.8; p = 0.0054) (Fig. 3B). We also examined the subclasses of IgG present in glomerular deposits (Fig. 3A). In B6lpr/lpr-Tlr4−/− mice, the intensity score of IgG1, IgG2a, IgG2b, and IgG3 deposits was decreased as compared with B6lpr/lpr mice, but the difference for each subclass was not significant (Fig. 3B).

ANOVA in TLR2- and TLR4-deficient B6lpr/lpr mice

The B6lpr/lpr mouse strain develops a lupus-like syndrome marked by a characteristic autoantibody response, in particular directed against DNA (30). To investigate the role of TLR2 and TLR4 in the production of autoantibodies, sera from 6-month-old B6lpr/lpr, Tlr2−/−, B6lpr/lpr-Tlr4−/−, and B6lpr/lpr mice were examined by indirect immunofluorescence analysis using HEp-2 cells and intensity scores from 0 to 4 were established as previously described (29). Sera from B6lpr/lpr-Tlr2−/− and B6lpr/lpr-Tlr4−/− mice exhibited a homogenous staining of the nucleus as B6lpr/lpr mouse sera but the fluorescence intensity was dramatically diminished, particularly in B6lpr/lpr-Tlr4−/− mice (mean of score = 1.7 ± 1.1) vs B6lpr/lpr mice (3.8 ± 1; p = 0.042) (Fig. 4A). Next, we assessed the IgG subclasses of ANA (Fig. 4B). B6lpr/lpr-Tlr2−/− and B6lpr/lpr-Tlr4−/− mice sera presented a diminution of IgG2a staining vs B6lpr/lpr mouse (p = 0.049 and p = 0.037, respectively).

Autoantibody specificities in TLR2- and TLR4-deficient B6lpr/lpr mice

Then, we investigated whether the reduced homogenous nuclear staining given by B6lpr/lpr-TLR-deficient mouse sera was accompanied by a quantitative change of the autoantibody specificities usually detected in B6lpr/lpr mice (Fig. 5A). Thus, we performed analyses of anti-dsDNA, ssDNA, and nucleosome Ab rates in WT (n = 10), TLR2 (n = 10), and TLR4-deficient B6lpr/lpr mice (n = 8). The rate of IgG anti-dsDNA and anti-ssDNA autoantibodies decreased significantly in B6lpr/lpr-Tlr2−/− (mean OD405 nm = 0.26 ± 0.04; p = 0.0002; 0.3 ± 0.078; p = 0.0007, respectively) and B6lpr/lpr-Tlr4−/− (0.27 ± 0.017; p = 0.0007; 0.28 ± 0.05; p = 0.0004, respectively) vs B6lpr/lpr mouse sera (0.43 ± 0.09; 0.48 ± 0.043, respectively). In contrast, the production of IgG
anti-nucleosome Abs was unaffected in B6<sup>lpr/lpr-Tlr4<sup>-/-</sup></sup> mice but decreased in B6<sup>lpr/lpr-Tlr2<sup>-/-</sup></sup> mice. To determine whether the difference in anti-DNA Ab titers observed at 6 mo of age could be related to a delayed appearance of autoantibodies, a kinetic analysis of anti-nucleosome and anti-dsDNA Abs in B6<sup>lpr/lpr-Tlr4<sup>-/-</sup></sup> mice was performed from 2 to 5 mo of age (Fig. 5B). Anti-nucleosome Abs were absent in B6<sup>lpr/lpr-Tlr4<sup>-/-</sup></sup> at 2 mo of age (<i>p</i> = 0.0001), but increased from 3 mo and reached a level similar to that observed in B6<sup>lpr/lpr</sup> mice at 5 mo. Conversely, anti-dsDNA Ab levels, which were also lower in B6<sup>lpr/lpr-Tlr2<sup>-/-</sup></sup> than in B6<sup>lpr/lpr</sup> at 2 mo of age, did not increase significantly after 3 mo and did not reach the level observed in B6<sup>lpr/lpr</sup> mice (Fig. 5B).

We also examined the response against cardiolipin, another structurally repetitive Ag, and observed that the rate of IgG anti-CL Abs was also significantly lower in TLR2- and TLR4-deficient B6<sup>lpr/lpr</sup> than in B6<sup>lpr/lpr</sup> mouse sera (respectively, 0.35 ± 0.2 vs 0.91 ± 0.21, <i>p</i> = 0.0007; 0.35 ± 0.2 vs 0.91 ± 0.21).

RF and antibacterial autoantibody production in TLR2- and TLR4-deficient B6<sup>lpr/lpr</sup> mice

The antibacterial repertoire of IgM Abs and RF was analyzed by Western blotting using <i>E. coli</i> (Fig. 6A) and <i>S. aureus</i> (Fig. 6B) protein extracts as substrates and ELISA, respectively. RF levels were decreased in both TLR2- and TLR4-deficient mice at 6 mo of age (0.36 ± 0.22 and 0.2 ± 0.04, <i>p</i> = 0.0003, respectively) in comparison to B6<sup>lpr/lpr</sup> mice (0.54 ± 0.37) (Fig. 5A). Similarly, the reactivity of B6<sup>lpr/lpr-Tlr4<sup>-/-</sup></sup> sera with both bacterial protein extracts was clearly diminished in comparison to B6<sup>lpr/lpr</sup> mice and showed a similar pattern to that of normal B6 mice (Fig. 6, A and B).
Ig levels in mice

Since most rates of autoantibody decreased in TLR2- and TLR4-deficient mice, we analyzed the serum Ig level in the different groups of mice. Although the total IgG level was not significantly diminished in B6<sup>lpr/lpr</sup>-TLR-deficient mice, the distribution of IgG subclasses was modified (Fig. 7). Indeed, IgG2a levels were significantly lower in B6<sup>lpr/lpr</sup>-Tlr4<sup>-/-</sup> (0.119 ± 0.03, p = 0.004) than in B6<sup>lpr/lpr</sup> mice (0.351 ± 0.12). In B6<sup>lpr/lpr</sup>-TLR2<sup>-/-</sup> mice, the IgG2a subclass also decreased. In both TLR-deficient mice, IgG1 and IgG2b were rather increased as compared with B6<sup>lpr/lpr</sup>. We also compared the level of IgM and found no significant difference between the three groups of mice (Fig. 6C).

Cytokine profile in TLR2- and TLR4-deficient B6<sup>lpr/lpr</sup> mice

Dysregulation of cytokine profiles is another feature of lpr-induced SLE. Glomerulonephritis in B6<sup>lpr/lpr</sup> mice is associated with a predominance of Th1 cells producing high level of IFN-γ (31). To assess the effects of TLR2 and TLR4 deficiency on Th1/Th2 balance, plasma levels of IL-6, IL-10, IL-12, IL-13, and IFN-γ were measured using the Luminex technology (Fig. 8). The IFN-γ level was dramatically decreased in B6<sup>lpr/lpr</sup>-TLR4<sup>-/-</sup> (70.8 ± 113 pg/ml; p = 0.0005) vs B6<sup>lpr/lpr</sup> mice.
No difference was observed in IL-12 and IL-13 production. IL-10 values were highly scattered in B6<sup>−/−</sup>-TLR2<sup>−/−</sup> mice and were rather decreased in B6<sup>−/−</sup>-TLR4<sup>−/−</sup> mice. Interestingly, IL-6 production was clearly decreased in B6<sup>−/−</sup>-TLR4<sup>−/−</sup> (481.3 ± 465 pg/ml; p = 0.03) comparatively to B6<sup>−/−</sup> mice (Fig. 8). We confirmed this result by evaluating the level of IL-6 secretion from splenocytes and purified B cells after 24 h in vitro culture in medium (data not shown).

B cell subsets in TLR2- and TLR4-deficient B6<sup>−/−</sup> mice

Flow cytometric analysis of spleen cells from TLR2- or TLR4-deficient mice showed a normal composition of lymphocyte populations (15, 32). Flow cytometry analysis of B cell lineage in bone marrow from TLR2- and TLR4-deficient mice (33), but also from B6<sup>−/−</sup>, B6<sup>−/−</sup>-TLR2<sup>−/−</sup>, and B6<sup>−/−</sup>-TLR4<sup>−/−</sup> showed no significant abnormality in the pre-B, immature B, or mature B cell populations (data not shown). To explain the decrease of autoantibody production directed against T-independent Ags, including DNA, cardiolipin, and RF in TLR-deficient mice, we analyzed splenic B cell subsets in B6<sup>−/−</sup>-TLR-deficient mice. Interestingly, we showed that percentages of the marginal zone (MZ) B cell population (IgM<sup>high</sup>IgD<sup>low</sup>CD21<sup>+</sup>) were lower in B6<sup>−/−</sup>-TLR2<sup>−/−</sup> (1.8 ± 1.1; p = 0.023) and B6<sup>−/−</sup>-TLR4<sup>−/−</sup> (1.7 ± 0.5; p = 0.004) than in B6<sup>−/−</sup> mice (5.4 ± 1.6) (Fig. 9B and Table I). The decrease of MZ cells was not correlated with an increase of a follicular B cell population. Because these cells are also characterized by CD23 expression, we performed FACS analyses using CD19, CD21, and CD23 markers. Interestingly, in B6<sup>−/−</sup>-TLR4<sup>−/−</sup> mice, the expression of CD23 strongly diminished on all B cells as shown by the mean fluorescence intensity which was of 3.3 ± 0.9, 3 ± 0.6, and 1.8 ± 0.6 in B6<sup>−/−</sup>, B6<sup>−/−</sup>-TLR2<sup>−/−</sup>, and B6<sup>−/−</sup>-TLR4<sup>−/−</sup>, respectively (Fig. 10).

Discussion

Our study shows that TLR4- and, at a lesser extent, TLR2-deficient B6<sup>−/−</sup> mice develop a significantly less severe lupus-like disease than B6<sup>−/−</sup>, as demonstrated by the reduced intensity of glomerulonephritis and the decrease of autoantibody rates.

These results are in accordance with previous studies which demonstrated that the injection of bacterial LPS in mice is able to accelerate and induce lupus manifestations. Indeed, LPS, a major
TLR4 ligand, accelerates the disease when administered into BW mice and induces anti-ssDNA and anti-dsDNA Abs and IgG glo-
merular deposits in normal mice (34). In addition, transgenic mice that constitutively expressed the chaperone molecule gp96 and 
consequently overexpressed TLR4 on the cell surface developed a lupus-like disease probably due to an enforced TLR4 response to 
LPS since overexpression of the \textit{Tlr4} gene alone was sufficient to 
induce the disease (26). Our strategy which consisted in the inac-
tivation of \textit{Tlr4} in \textit{B6 lpr/lpr} mice provides complementary and de-
finite data showing the role of TLR4 in the development of lupus 
in mice.

Several observations reported in the present article allow us to 
propose different but not exclusive mechanisms accounting for the 
phenotypic changes induced by the inactivation of TLR2 or TLR4. 
First, the analysis of the Ab response in TLR2- and TLR4-deficient 
\textit{B6 lpr/lpr} mice showed a significant diminution of autoantibody 
rates, notably directed against DNA and cardiolipin, two highly 
repetitive structures considered as T cell- independent (TI) Ags. In 
contrast, the Ab response directed against nucleosome-derived 
proteins was either poorly affected or even unaffected. TI Ags can 
mediate cross-linking of B cell Ag receptors in a multivalent fash-
ion and activate specific B cells in the absence of T cells (35).

Nevertheless, these B cells are thought to require a second CD4^{+} 
TI signal which may be delivered from various origins (36), not-
ably from specific components of pathogens able to induce en-
gagement of TLR expressed by B and dendritic cells (37, 38). In 
this regard, LPS, the major component of the cell walls of Gram-
negative bacteria, has been shown to mediate an enhancing effect 
on the TI Ab responses (39) and to induce IgM secretion in a 
TLR4-dependent process (40). Similarly, ligands of TLR2 could 
provide synergistic signals participating in the activation, prolif-
eration, and production of Abs by B cells (41). Thus, it is expected 
that the lack of TLR2 or TLR4 expression on B and dendritic cells 
is accompanied by the diminution of the autoantibody response to 
TI Ags. Indeed, the reactivity of sera from \textit{B6 lpr/lpr--Tlr4-deficient} 
mice was strongly decreased against both Gram-positive and 
Gram-negative bacterial extracts. Thus, the absence of a second 
signal may explain, on the one hand, the decreased production of 
anti-DNA and anti-CL Abs and, on the other hand, the unchanged 
anti-nucleosome autoantibody response considered to be depen-
dent on either the presence of T cell clones specific for nucleosome 
(42) or TLR9 signaling (20, 21).

It is well established that the response against TI Ags involves 
MZ or B1 B lymphocytes (43). Consequently, the dramatic changes of autoantibodies directed against DNA, CL, and RF 

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<th>\textbf{Table I.} \textit{B cell subset distribution in B6\textit{ lpr/lpr}, B6\textit{ lpr/lpr--Tlr2--}, and B6\textit{ lpr/lpr--Tlr4--} mice (mean ± SD)}^a</th>
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<tr>
<td><strong>T1</strong></td>
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<tr>
<td>IgM^{hi}IgD^{lo/hi}CD21^{+}</td>
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<tr>
<td>\textit{B6 lpr/lpr} (\textit{n = 13})</td>
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<tr>
<td>\textit{B6 lpr/lpr--Tlr2--} (\textit{n = 11})</td>
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<td>\textit{B6 lpr/lpr--Tlr4--} (\textit{n = 14})</td>
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^a Percentages of B cell subsets in gated CD19^{+} splenocytes of 6-mo-old mice were determined by flow cytometry according to IgM, IgD, and CD21 expression and compared by using the Mann-Whitney \textit{U} test (*, \textit{p} = 0.023 and **, \textit{p} = 0.004).
prompted us to study the distribution of the different B cell populations present in TLR-deficient mice. The major observation is that TLR2- and 4-deficient B6*lp/lp* mice exhibit a lower proportion of MZ B cells than B6*lp/lp* mice that could constitute another factor contributing to the decreased production of autoantibodies directed against TI Ags. Indeed, several works have shown that the autoantibody response, notably in MRL-Fas*lp/lp* mice, does not occur in the germinal centers but takes place at extrafollicular sites similar to those involving TI responses (44). This is particularly demonstrated for RF-secreting B cells and highly suspected for DNA-specific B cells (45, 46). Thus, the present observation strongly supports the view that lupus-related autoreactive B cell responses involve extrafollicular pathways. Incidentally, the present article also provides information on the development and maintenance of MZ B cells. First, two subsets of transitional B cells, termed T1 and T2, have been described (47). They are separated among IgM*high* splenic B cells by differential expression of IgD, CD21, CD23, and AA4.1: T1 being (IgD*/low*CD21*/low*CD23*/low*AA4.1*+) and T2 (IgD*high*CD21*/high*CD23*/high*AA4.1*+) (48). Most models suggest that T2 cells derive from T1 cells and seed both the follicular (CD21*/low*CD23*/low*) and MZ (CD21*/high*CD23*/low*) B cell compartment. Since CD23 is found on the surface of T2 and follicular B cells but not on MZ cells (48), it has been suggested that CD23 was lost at the MZ stage, probably due to its cleavage from the cell surface (49). A recent alternative interpretation is that the MZ-specific lineage never expresses CD23 and directly derives from T1 cells (50). In our models, the parallel decrease of T1 cells and MZ B cells argues for this hypothesis. Another feature of B lymphocytes derived from B6*lp/lp*-TLR4-deficient mice is the lack of CD23. In this regard, in vitro experiments have demonstrated that CD23 expression can be induced on B cells by TLR4 engagement during late steps of B lymphocyte maturation (33). The low level of CD23, observed on cells from our B6*lp/lp*-TLR4-deficient mice is in agreement with this role of TLR4 in the differentiation program of B cells and the expression of CD23 on late steps of B lymphocyte maturation. It could be noted that the absence of CD23 does not affect the development of T2 and mature B cells since these B cell populations are detected in our animals using other markers.

Second, the reduced MZ B cell number in TLR4-deficient mice could be related to the role of TLR stimulation on the B lymphocyte stimulator (BlyS) receptor expression. Indeed, the stimulation of TLR4 with LPS increases the BlyS-binding capacity by strongly up-regulating the transmembrane activator calcium modulator and cyclophilin ligand (TACI), in particular on MZ B cells (51). BlyS, also called BAFF, and its receptors are well known to mediate survival of peripheral immature B lymphocytes (52) and to profoundly influence B cell homeostasis (53). The number of MZ B cells is increased in BAFF-transgenic (54) as well as TACI-deficient (55) mice, two models in which an increase of MZ B cell is associated with an increase of autoantibody production. Moreover, the blockage of BAFF prolongs the survival of NZB/W mice and prevents glomerulonephritis and kidney inflammation (56).
Our results also showed a significant decrease of IL-6 and IFN-γ in TLR4-deficient B6/lpr/lpr mice, two cytokines previously shown to participate in the production of lupus-related autoantibodies and lupus disease. IL-6 is a multifunctional cytokine that is critical for B cell cell differentiation and maturation, Ig secretion, and renal mesangial proliferation (57). Overproduction of IL-6 has been associated with SLE (58) and IL-6 administration exacerbated the lupus disease in NZB/W F1 mice (59). Conversely, anti-IL-6 mAb inhibited autoimmune responses in this lupus strain (60) and had a beneficial effect on the renal disease by inducing a diminution of anti-dsDNA autoantibody production and a significant reduction of the number of CD21⁺ CD23⁻ MZ B cells, all features that were also present in TLR4-deficient B6/lpr/lpr mice. Similarly, IFN-γ is involved in the pathogenesis of mouse lupus (61–63). IFN-γ/−/− MRL mice produced reduced titers of IgG2a and IgG2b autoantibodies and developed less severe lymphadenopathy and tissue injury (64). Thus, a third mechanism through which TLR2 and TLR4 may play a role in the pathogenesis of lupus in B6/lpr/lpr mice is their role in the pattern of cytokine secretion.

Taken together, our results clearly show that TLR4 and at a lesser extent TLR2 participate in the development of lupus. These TLRs could act on either the onset of the disease or its progression and be activated by exogenous or endogenous ligands. TLR stimulation could be triggered by microbial infection and induce the production of pathogenic autoantibodies at extrafollicular sites, in particular by MZ B cells (65). Cellular injury could constitute another source of molecules able to stimulate TLR: HSP60 (66) and HSP70 (67) have been described as TLR2 and 4 ligands; fibronectin and hyaluronate that are released from cells in response to tissue damage were shown to activate TLR4 (68, 69); high-mobility group box 1, originally described as a DNA-binding protein, can also be released extracellularly and interact with TLR2 and TLR4, generating inflammatory responses similar to those initiated by LPS (70).

In conclusion, this study shows that TLR2 and TLR4 modulate the autoimmune manifestations of SLE by participating in pathogenic autoantibody production and tissue injury through different cellular and molecular mechanisms. These findings provide new insights into the mechanisms controlling the relationship between innate immunity and the adaptive response that characterized a genetic autoantibody production and tissue injury through different cellular and molecular mechanisms. These findings provide new insights into the mechanisms controlling the relationship between innate immunity and the autoimmune manifestations of SLE by participating in patho-

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Disclosures

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